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# Antioxidant and Antimelanogenic Activities of Polyamine Conjugates from Corn Bran and Related Hydroxycinnamic Acids

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The antioxidant activity of three major polyamine conjugates, *N*,*N*′-dicoumaroyl-putrescine (DCP), *N*-*p*-coumaroyl-*N*′-feruloylputrescine (CFP), and *N*,*N*′-diferuloyl-putrescine (DFP) isolated from corn bran, and their related hydroxycinnamic acids, *p*-coumaric acid and ferulic acid, were evaluated by three antioxidant in vitro assay systems, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and superoxide and hydroxyl radicals generated by enzymatic and nonenzymatic reactions. Additionally, five phenolic compounds were evaluated for melanogenesis inhibitory activity using mushroom tyrosinase and B16 melanoma cells. Most of the phenolic compounds significantly scavenged DPPH, superoxide, and hydroxyl radicals in a dose-dependent manner. Particularly, DFP showed potent DPPH ($IC_{50}$ = 38.46 $\mu$M) and superoxide ($IC_{50}$ = 291.62 $\mu$M) radical scavenging activities, while DCP exhibited the strongest hydroxyl radical scavenging activity ($IC_{50}$ = 120.55 $\mu$M). CFP also exerted moderate DPPH, superoxide, and hydroxyl radical scavenging activities. Meanwhile, DCP ($IC_{50}$ = 181.73 $\mu$M) showed potent tyrosinase inhibitory activity toward L-tyrosine as the substrate, whereas DFP ($IC_{50}$ = 733.64 $\mu$M) significantly inhibited melanin synthesis in B16 melanoma cells. These current results indicate that these three polyamine conjugates from corn bran may be useful potential sources of natural antioxidants and skin-whitening agents.

**KEYWORDS: Corn bran; polyamine conjugates; hydroxycinnamic acids, active oxygen species; antioxidants; tyrosinase; melanin synthesis**

## INTRODUCTION

Recently, much attention has been focused on dietary natural antioxidants capable of inhibiting reactive oxygen radical-mediated oxidative stress, which is involved in several pathological diseases, such as cancer, atherosclerosis, diabetes, inflammation, and aging (*1*, *2*). Particularly, superoxide and hydroxyl radicals of reactive oxygen species are known to cause severe oxidative damage to susceptible biomolecules, thereby eventually contributing to deleterious biological effects, including carcinogenesis, inflammation, mutagenesis, and cytotoxicity (*3*, *4*). For these reasons, an extensive search for novel natural antioxidants acting as radical scavenger was undertaken.

Naturally occurring hydroxycinnamic acid derivatives, such as *p*-coumaric acid (CA), ferulic acid (FA), and caffeic acids, are well-known to have a variety of biological activities, such as anticancer (*5*), antiinflammation (*5*), antihepatoxicity (*6*), antibacterial (*7*), antimutation (*8*), and antioxidation (*9*, *10*). Particularly, several hydroxycinnamic acids and their derivatives have been found to possess strong antioxidant activities as radical scavengers against reactive oxygen species (*11*−*14*) and to have considerable tyrosinase inhibitory activities (*15*, *16*). However, information on reactive oxygen radical scavenging activities of phenolic acid amides containing a parent moiety of CAs and FAs and on their antimelanogenic activity is still very limited.

Corn bran, which is an important byproduct of the corn dry-milling industry, is rich in several functional lipid constituents including unsaturated fatty acids, tocopherols and phytosterols, and dietary fibers and carotenoid pigments, etc. (*17*). Also, corn bran has recently received a renewed interest (*18*) as a functional phytochemical source due to its high levels of FAs and CAs, their (dehydro) dimers and trimers, and their polyamine conjugates, such as diferuloylputrescine (DFP), *p*-coumaroyl-

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feruloylputrescine (CFP), and dicoumaroylputrescine (DCP). DFP and CFP exhibit many biological activities, including antidiuretic (*19*), inhibition of aflatoxin biosynthesis (*20*), and antioxidant activities (*21*−*24*). We are unaware of any studies that report the radical scavenging activity of corn-derived polyamine conjugates against reactive oxygen species and the antimelanogenic activity against tyrosinase and melanoma cells.

The purpose of the present study is to evaluate the radical scavenging activity of three polyamine conjugates, DCP, DFP, and CFP, isolated from corn bran, and two related hydroxycinnamic acids, CAs and FAs, against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, superoxide anion induced by xanthine−xanthine oxidase, and hydroxyl radical generated via the Fenton reaction. Additionally, the antimelanogenic activity of five phenolic compounds was also determined by in vitro assays using mushroom tyrosinase and B16 melanoma cells.

## MATERIALS AND METHODS

**Materials.** Corn bran (Dietfiber Ultrafine Corn Bran, NU 20085) was kindly provided by Bunge North America (Danville, IL). All of the corn bran used was from the same lot, which was received in June 2005 and stored at 4 °C.

**Chemicals.** CAs and FAs, DPPH, xanthine oxidase (EC 1.2.3.2), xanthine, nitrobluetetrazolium chloride (NBT), thiobarbituric acid (TBA), $H_2O_2$, 2-deoxyribose, bovine serum albumin (BSA), trifluoroacetic acid (TFA), mushroom tyrosinase (EC 1.14.18.1), L-tyrosine, L-3,4-dihydroxyphenylalanine (L-DOPA), kojic acid, arbutin, and NMR solvents were obtained from Sigma Chemical Co. (St. Louis, MO). L-Ascorbic acid, $FeCl_3 \cdot 6H_2O$, and ethylendediaminetetraacetic acid (EDTA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Potassium hydroxide and potassium phosphate monobasic were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). All solvents for high-performance liquid chromatography (HPLC) analysis were of Merck HPLC grade. All other reagents used in this study were of analytical grade.

**Isolation and Purification of Polyamine Conjugates from Corn Bran.** Corn bran powder (2.0 kg) was defatted with *n*-hexane at ambient temperature in an ultasonicator, and the defatted residue was extracted continuously with 80% aqueous ethanol (40 L) in an ultrasonicator, then filtered, and evaporated under reduced pressure. The EtOH extract (65.6 g) was redissolved in 80% aqueous MeOH and washed twice with *n*-hexane to remove lipids and pigments. The lower layer was evaporated to small volume and partitioned successively with dichloromethane (10 L), ethylacetate (10 L), and *n*-butanol (10 L). The $CH_2Cl_2$ fraction (4.92 g), EtOAc fraction (1.44 g), and *n*-BuOH fraction (9.34 g) were evaluated for antioxidant activity using a DPPH radical. Among three fractions, the strongest antioxidant $CH_2Cl_2$ fraction (4.92 g) was chromatographed on a silica gel column (5.0 cm × 30 cm) with *n*-hexane−EtOAc−MeOH−TFA (200:200:100:1, v/v) as an eluent, to afford 17 fractions: Fr. 1 (154.7 mg), Fr. 2 (30.1 mg), Fr. 3 (53.2 mg), Fr. 4 (82.2 mg), Fr. 5 (107.2 mg), Fr. 6 (91.7 mg), Fr. 7 (56.7 mg), Fr. 8 (77.6 mg), Fr. 9 (60.5 mg), Fr. 10 (31.3 mg), Fr. 11 (28.4 mg), Fr. 12 (16.6 mg), Fr. 13 (34.5 mg), Fr. 14 (67.9 mg), Fr. 15 (57.0 mg), Fr. 16 (216.5 mg), and Fr. 17 (21.5 mg). Fraction 13 (34.5mg) was further chromatographed on a ODS-A column (2.0 cm × 30 cm) chromatography with 60% aqueous MeOH, and three fractions were obtained as follows: Fr. 1 (4.2 mg), Fr. 2 (20.4 mg), and Fr. 3 (3.1 mg). Fraction 2 was chromatographed on a Sephadex LH-20 column (2.0 cm × 80 cm) with 80% aqueous MeOH, to separate a white pure DCP (13.6 mg, 0.68 mg %, $R_t$ = 28 min for reversed-phase HPLC). Fraction 14 (67.9 mg) was also chromatographed on a ODS-A column (2.0 cm × 30 cm) with 60% MeOH, and three fractions were obtained as follows: Fr. 1 (8.3 mg), Fr. 2 (35.7 mg), and Fr. 3 (11.3 mg). Fraction 2 was rechromatographed on a Sephadex LH-20 column (2.0 cm × 80 cm) with 80% MeOH and yielded a pure CFP (28.8 mg, 1.44 mg %, $R_t$ = 30 min for reversed-phase HPLC). Finally, the same purification procedure on ODS-A and Sephadex LH-20 column chromatographies was carried out successively for Fr. 16 (216.5 mg), and a pure white DFP powder (156.3 mg, 7.82 mg %, $R_t$ = 32 min for reversed-phase HPLC) was separated.

The purified compounds as described above were characterized by UV/vis, infrared (IR), liquid chromatography−mass spectrometry (LC-MS), and NMR analyses. UV and IR spectra were recorded on an Jasco 3334 UV/vis spectrophotometer (Osaka, Japan) and a FT-IR spectrometer (IFS 120 HR, Bruker, Germany). $^1$H NMR (400 MHz) and $^{13}$C NMR (100 MHz) spectra were measured in dimethyl sulfoxide (DMSO)-$d_6$ on a Unity Plus 300 spectrometer (Varian, United States), and chemical shifts are given as $\delta$ values with tetramethylsilane as an internal standard. Fast-atom bombardment mass spectrometry (FAB-MS) was performed on a JMS-700 mass spectrometer (ion source, Xe atom beam; accelerating voltage, 10 kV; JEOL, Japan) using *m*-butylalcohol as a mounting matrix. Identification of three polyamine conjugates isolated from the $CH_2Cl_2$ fraction of corn bran by a silica gel column chromatography as described above was carried out a reversed-phase HPLC-ELSD method using a Hewlett-Packard model 1100 HPLC, with autosampler and detection by both an HP model 1100 diode array UV−visible detector (Agilent Technologies, Avondale, PA) and a Sedex model 55 evaporative light scattering detector (Richard Scientific, Novato, CA), operated at 40 °C with a nitrogen gas pressure of 2 bar. HPLC analysis was carried out using a Prevail $C_{18}$ column (2.1 mm i.d. × 150 mm, Alltech Associates Inc., Deerfield, IL) with a linear gradient from $H_2O$ (solvent A) to 0.2% v/v $CH_3COOH$ in MeOH (solvent B) for 60 min at a flow rate of 0.2 mL/min. The elution profile was as follows: 0−20 min, 60% A, 40% B; 40−50 min, 0% A, 100% B; 51−60 min, 60% A, 40% B. Column chromatography was performed on silica gel (230−400 mesh, Merck, Darmstadt, Germany), ODS-A gel (12 nm, 150 $\mu$m, YMC, Inc., MA), and Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden). All solvents used for this study were of analytical and HPLC grades.

**DPPH, Superoxide, and Hydroxyl Radical Scavenging Activity.** Radical scavenging activities of three polyamine conjugates and CAs and FAs on DPPH, superoxide, and hydroxyl radicals were determined as previously reported (*25*). In the assay for DPPH radical scavenging, each phenolic compound was added to 100 $\mu$M DPPH in methanol, followed by incubation at 25 °C. Their reactivity with DPPH was determined spectrophotometrically at 516 nm.

In the assay for the superoxide radical scavenging, the reaction mixture (3.0 mL), comprising 0.05 M $Na_2CO_3$ buffer (pH 10.2) containing 3 mM xanthine, 3 mM EDTA, BSA (1.5 mg protein/mL), 0.75 mM NBT, test solution (1−300 mM in DMSO), and 5.0 U/mL xanthine oxidase was incubated at 25 °C for 20 min, and then, the reaction was terminated by the addition of 6 mM $CuCl_2$. The production of formazan was determined at 560 nm.

In the assay for the hydroxyl radical scavenging, the reaction mixture (1.0 mL), comprising 30 mM $KH_2PO_4$−KOH buffer (pH 7.4), 2 mM 2-deoxyribose, 0.1 mM $FeCl_3 \cdot 6H_2O$, 104 $\mu$M EDTA, test solution (1−10 mM in above buffer), 1.0 mM $H_2O_2$, and 0.1 mM L-ascorbic acid, was incubated at 37 °C for 1 h. The thiobarbituric acid reactive substance level was determined after terminating the reaction by the addition of TBA reagent. $IC_{50}$ values against three radical scavenging activities were determined by regression analysis of the results obtained at three different concentrations of the sample.

**Tyrosinase Assay.** The tyrosinase assay was performed by the slightly modified method of Masamoto et al. (*26*) using L-tyrosine or L-DOPA as the substrates. The reaction mixture (1.55 mL) using L-tyrosine as the substrate contained 0.5 mL of 0.1 M phosphate buffer (pH 6.8), 0.45 mL of deionized water, 0.05 mL of the samples solution at various concentrations in DMSO, and 0.05 mL of tyrosinase (2000 U/mL) and was preincubated at 25 °C for 5 min before 0.5 mL of 2.5 mM L-tyrosine was added. The mixture was preincubated in a water bath at 37 °C for 5 min and stopped with cold ice for 5 min. The reaction was monitored at 475 nm for 2 min. A control reaction was carried out without the test sample. The O.D. values were measured by a UV spectrophotometer (Jasco, Japan) at 475 nm. The percentage inhibition of tyrosinase was calculated as follows: inhibition (%) = (A − B)/A × 100, where A and B represent the absorbance of test solution and simultaneous control, respectively.

Similarly, the reaction mixture (3 mL) using L-DOPA as the substrate contained 1 mL of 1.5 mM L-DOPA solution, 0.1 mL of DMSO with

**Table 1.** DPPH Radical Scavenging Activity of the Crude Ethanol Extract and Three Solvent Fractions from Corn Bran[a]

| extract and fraction | DPPH radical scavenging activity ($IC_{50}$, $\mu$g/mL) |
|---|---|
| crude ethanol extract | 79.4 ± 2.9 |
| dichloromethane fraction | 41.9 ± 1.7 |
| ethylacetate fraction | 51.6 ± 2.7 |
| *n*-butanol fraction | 161.1 ± 2.9 |

[a] Data represent means ± SD of triplicate determination. All values in column are significantly different at $P < 0.05$.

or without a sample, and 1.8 mL of 1/15 M phosphate buffer solution (pH 6.8) and was incubated at 25 °C for 10 min. Furthermore, 0.1 mL of 1000 units/mL tyrosinase in aqueous solution was added to the mixture to immediately measure the initial rate of linear increase in optical density at 475 nm. A control reaction was conducted with DMSO. Tyrosinase inhibition was calculated as described above. Kojic acid was used as a reference.

**Inhibition of Melanin Production in B16 Melanoma Cells.** Melanin biosynthesis was assayed by the method of Gordon et al. (*27*) as modified by Roh et al. (*33*). Melanoma cells, B16, strain C57BL/6J, were purchased from the KCLB (Korean Cell Line Bank, Daejon, Korea) and grown in a humidified atmosphere with 5% $CO_2$ at 37 °C. Cells ($1 \times 10^5$) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, United States) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotic−antimycotic (Gibco BRL Co., NY). After 24 h of cultivation, the medium was changed with new DMEM medium containing test samples of various concentrations. After 2−3 days of incubation, the adherent cells were washed with phosphate-buffered saline (PBS) and detached by trypsinization. The cells were lysed with 10% DMSO/1 N NaOH and stored at room temperature for 10 min. After centrifugation at 3000 rpm for 10 min, the melanin content was determined at 490 nm using the ELISA Microplate Reader (EL800, Bio-Tex Instruments, Inc., United States).

**Statistical Analysis.** All experiments were performed in three replicates. In particular, triplicate analyses of a single extraction were used for antioxidant and antimelanogenic assays of three isolated polyamine conjugates. A curve of the concentration plotted against the percentage inhibition was used to calculate the half-maximal inhibition concentration ($IC_{50}$). Statistical analysis was performed using Duncan's multiple range test (*28*).

## RESULTS AND DISCUSSION

**DPPH Radical Scavenging Activity of the Crude EtOH Extract and Its Solvent Fractions from Corn Bran.** DPPH radical scavenging activities (RSA) of crude EtOH extract and its three solvent fractions ($CH_2Cl_2$, EtOAc, and *n*-BuOH fractions) from corn bran were determined to search for fractions with high antioxidant activities. The ethanol and three solvent extracts showed significant RSA in a dose-dependent manner (data not shown). The 50% inhibitory concentration ($IC_{50}$) of each extracts was calculated from the results. As shown in **Table 1**, the crude EtOH extract from corn bran exhibited significant DPPH RSA with an $IC_{50}$ value of 79.43 $\mu$g/mL. Among three solvent fractions, the $CH_2Cl_2$ fraction exhibited the strongest DPPH RSA ($IC_{50} = 41.89$ $\mu$g/mL), followed by the EtOAc fraction ($IC_{50} = 51.64$ $\mu$g/mL) and the *n*-BuOH fraction ($IC_{50} = 161.11$ $\mu$g/mL), in descending order. Herein, we have isolated three major polyamine conjugates from the strong antioxidant $CH_2Cl_2$ fraction, as described in the Materials and Methods.

**Structural Elucidation of Three Polyamine Conjugates.** The chemical structures of three polyamine conjugates, DCP, CFP, and DFP, have been assigned on the basis of their UV, IR, NMR, and FABMS spectral data and comparison with published data (*19*). Three polyamine conjugates were first elucidated fully by $^1$H and $^{13}$C NMR and MS spectroscopy, even though three compounds have already been isolated and partially identified from corn (*18*, *19*). The detailed UV, IR, NMR, and FABMS spectral data of three polyamine conjugates are as follows.

*DFP*. Colorless white powder. UV $\lambda_{max}$ (MeOH) nm: 220, 234, 294, 320. IR $\nu_{max}$ (KBr) cm$^{-1}$: 3500 (NH, OH), 2927, 1652 (−CO−NH−), 1516−1484 (aromatic C═C), 1274. FABMS (positive ion) *m*/*z*: 441 $[M + H]^+$. $^1$H NMR (DMSO-$d_6$, 400 MHz, ppm): $\delta$ 9.24 (2H, *br s*, OH), 7.80 (2H, *t*, $J = 5.1$ Hz, NH), 7.18 (2H, *d*, $J = 15.7$ Hz, H-8 and H-8′), 6.94 (2H, *d*, $J = 1.8$ Hz, H-2 and H-2′), 6.81 (2H, *dd*, $J = 8.1$, 1.8 Hz, H-6 and H-6′), 6.62 (2H, *d*, $J = 8.1$ Hz, H-5 and H-5′), 6.26 (2H, *d*, $J = 15.7$ Hz, H-7 and H-7′), 3.63 (6H, *s*, 2 × $OCH_3$), 3.16 (4H, *m*, H-9 and H-9′), 1.30 (4H, *m*, H-10 and H-10′). $^{13}$C NMR (DMSO-$d_6$, 75 MHz, ppm): $\delta$ 165.60 (CO), 148.52 (C-4 and C-4′), 148.13 (C-3 and C-3′), 139.12 (C-7 and C-7′), 126.77 (C-1 and C-1′), 121.79 (C-6 and C-6′), 119.41 (C-8 and C-8′), 115.96 (C-5 and C-5′), 111.01 (C-2 and C-2′), 55.83 (2 × $OCH_3$), 48.68 (C-10 and C-10′), 27.14 (C-9 and C-9′).

*CFP*. Colorless white powder. UV $\lambda_{max}$ (MeOH) nm: 220, 229, 294, 310. IR $\nu_{max}$ (KBr) cm$^{-1}$: 3270 (NH, OH), 2930, 1655 (−CO−NH−), 1590−1510 (aromatic C═C), 1271. FABMS (positive ion) *m*/*z*: 411 $[M + H]^+$. $^1$H NMR (DMSO-$d_6$, 400 MHz, ppm): $\delta$ 9.67 (2H, *br s*, OH), 8.01 (2H, *t*, $J = 5.2$ Hz, NH), 7.40 (2H, *d*, $J = 8.6$ Hz, H-2 and H-6), 7.33 (2H, *d*, $J = 15.7$ Hz, H-8 and H-8′), 7.14 (1H, *d*, $J = 1.7$ Hz, H-2′), 7.00 (1H, *dd*, $J = 1.7$, 8.6 Hz, H-6′), 6.81 (1H, *d*, $J = 8.5$ Hz, H-5′), 6.48 (1H, *d*, $J = 8.5$ Hz, H-7 and H-7′), 6.42 (1H, *d*, $J = 15.2$ Hz, H-3 and H-5), 3.82 (3H, *s*, $OCH_3$), 3.20 (4H, *m*, H-9 and H-9′), 1.49 (4H, *m*, H-10 and H-10′). $^{13}$C NMR (DMSO-$d_6$, 75 MHz, ppm): $\delta$ 165.63 (CO), 159.13 (C-4), 148.55 (C-4′), 148.14 (C-3′), 139.14 (C-7), 138.86 (C-7′), 129.48 (C-2 and C-6), 126.76 (C-1′), 126.26 (C-1), 121.80 (C-6′), 119.39 (C-8), 119.10 (C-8′), 116.07 (C-5′), 115.97 (C-3 and C-5), 111.02 (C-2′), 55.83 ($OCH_3$), 48.94 (C-10), 38.69 (C-10′), 27.15 (C-9), 18.31 (C-9′).

*DCP*. Colorless white powder. UV $\lambda_{max}$ (MeOH) nm: 220, 229, 294, 310. IR $\nu_{max}$ (KBr) cm$^{-1}$: 3270 (NH, OH), 2930, 1655 (−CO−NH−), 1590−1510 (aromatic C═C), 1271. FABMS (positive ion) *m*/*z*: 381 $[M + H]^+$. $^1$H NMR (DMSO-$d_6$, 400 MHz, ppm): $\delta$ 9.82 (2H, *br s*, OH), 7.97 (2H, *t*, $J = 5.2$ Hz, NH), 7.38 (4H, *d*, $J = 8.4$ Hz, H-2 and H-2′, H-6 and H-6′), 7.30 (2H, *d*, $J = 15.6$ Hz, H-8 and H-8′), 6.78 (4H, *d*, $J = 8.8$ Hz, H-3 and H-3′, H-5 and H-5′), 6.38 (2H, *d*, $J = 15.6$ Hz, H-7 and H-7′), 3.16 (4H, *m*, H-9 and H-9′), 1.46 (4H, *m*, H-10 and H-10′). $^{13}$C NMR (DMSO-$d_6$, 75 MHz, ppm): $\delta$ 165.25 (CO), 158.73 (C-4 and C-4′), 138.46 (C-7 and C-7′), 129.10 (C-2 and C-2′, C-6 and C-6′), 125.92 (C-1 and C-1′), 118.76 (C-8 and C-8′), 115.69 (C-3 and C-3′, C-5 and C-5′), 38.32 (C-10 and C-10′), 26.79 (C-9 and C-9′).

**Radical Scavenging Activity of Three Polyamine Conjugates and Related Compounds.** Three polyamine conjugates, DCP, CFP, and DFP, isolated from the $CH_2Cl_2$ fraction of the corn bran, and their related hydroxycinnamic acids, CA and FA (chemical structures of five phenolic compounds were presented in **Figure 2**) were evaluated for their radical scavenging activity against DPPH radical, superoxide anion radical induced by xanthine−xanthine oxidase, and hydroxyl radical generated via the Fenton reaction, respectively. Five phenolic compounds exhibited concentration-dependent radical scavenging activities against the three radicals mentioned above (data

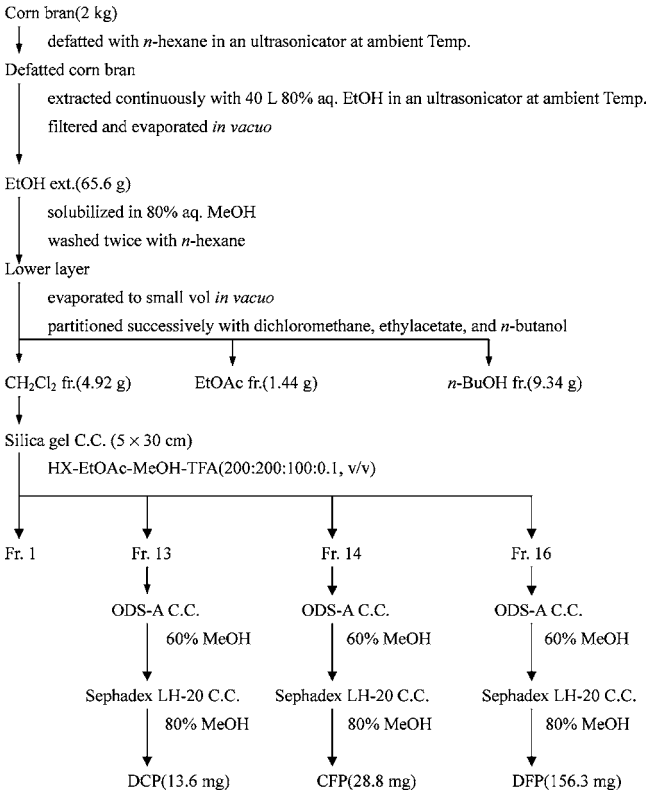


**Figure 1.** Schematic procedure for extraction, isolation, and purification of three polyamine conjugates from corn bran.

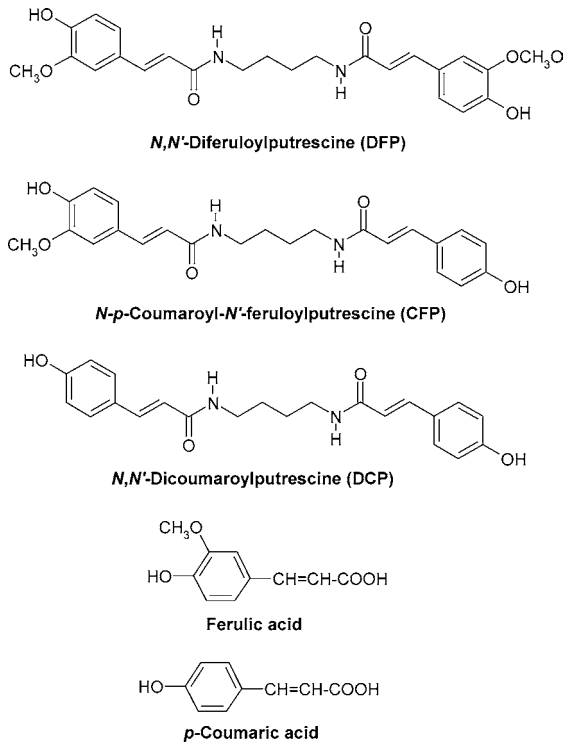


**Figure 2.** Chemical structures of three polyamine conjugates from corn bran and two hydroxycinnamic acids.

not shown), and the $IC_{50}$ values are listed in **Table 2**. Among five phenolic compounds, DFP ($IC_{50}$ = 38.46 $\mu$M) exerted a potent DPPH radical scavenging activity, followed by FA ($IC_{50}$ = 45.27 $\mu$M) > CFP ($IC_{50}$ = 70.37 $\mu$M), in decreasing order, but CA and DCP were less active. Thus, DFP exhibited considerable free radical scavenging activity as a hydrogen donor, and its activity was stronger than that of α-tocopherol ($IC_{50}$ = 52.34 $\mu$M), a well-known antioxidant ($P < 0.05$). Moreover, DFP ($IC_{50}$ = 291.62 $\mu$M) also showed the most potent superoxide anion radical scavenging activity, followed by CFP ($IC_{50}$ = 488.04 $\mu$M) > FA ($IC_{50}$ = 5312.30 $\mu$M), in decreasing order, but CA and DCP were less active. Thus, it should be noted that DFP showed potent DPPH and superoxide radical scavenging activities, and its DPPH and superoxide radical scavenging activities were stronger than that of α-tocopherol. Additionally, the order of superoxide radical scavenging activity of five phenolic compounds was very similar to that of their DDPH free radical scavenging activity, although there are some differences in the DPPH and superoxide radical scavenging activities of FA and CFP. Meanwhile, the hydroxyl radical scavenging activity of five phenolic compounds in decreasing order was DCP ($IC_{50}$ = 164.84 $\mu$M) > CFP ($IC_{50}$ = 192.45 $\mu$M) > CA ($IC_{50}$ = 256.31 $\mu$M) > DFP ($IC_{50}$ = 351.87 $\mu$M) > FA ($IC_{50}$ = 502.11 $\mu$M). The interesting phenomenon is that DCP exhibited the most potent hydroxyl radical scavenging activity, comparable to that of α-tocopherol ($IC_{50}$ = 155.67 $\mu$M) ($P < 0.05$). Thus, these results demonstrate that three polyamine conjugates in corn bran exhibit specific scavenging activities of the superoxide and hydroxyl radicals.

**Table 2.** Scavenging Activity ($IC_{50}$) of Three Major Polyamine Conjugates Isolated from Corn Bran and Their Related Compounds against DPPH, Superoxide, and Hydroxyl Radicals[a]

| | radical scavenging activity ($IC_{50}$, $\mu$M) | | |
|---|---|---|---|
| compound | DPPH radical | superoxide radical | hydroxyl radical |
| CA | >1000 | >20000 | 256.3 ± 11.9 c |
| FA | 45.3 ± 5.6 c | 5312.3 ± 260.0 a | 502.1 ± 21.1 a |
| CFP | 70.4 ± 0.5 a | 488.0 ± 20.0 b | 192.5 ± 14.0 d |
| DFP | 38.5 ± 2.3 c | 291.6 ± 4.8 c | 351.9 ± 13.4 b |
| DCP | >500 | >10000 | 164.8 ± 5.8 e |
| α-tocopherol[b] | 52.3 ± 0.4 b | >10000 | 155.7 ± 4.3 e |

[a] $IC_{50}$ represents the concentration of a compound required for 50% inhibition of DPPH radical, superoxide, and hydroxyl radicals. Data represent means ± SD of triplicate determination. Values with the different superscript letters in each column are significantly different at $P < 0.05$. [b] α-Tocopherol was used as a positive reference.

We next investigated some structure−activity relationships of five phenolic compounds. As shown in **Table 2**, DFP containing two FA and bisamide moieties showed stronger DPPH and superoxide anion radical scavenging activities than those of DCP containing two CA and bisamide moieties. These results support earlier reports that the DPPH and superoxide radical scavenging activities of the cinnamic acid derivatives with their moiety-containing phenolic amides increased with an increase in the number of hydroxyl groups and with the presence of a methoxyl group adjacent to the 4-hydroxyl group para-substituted on an aromatic ring (*29*, *30*), respectively. In contrast, of five phenolic compounds tested, DCP exhibited a potent hydroxyl radical scavenging activity, and the hydroxyl radical scavenging activity of CA and DCP containing two CA moieties was stronger than that of FA and DFP, respectively. These results support an earlier report that methoxylation of hydroxyl groups at the o-position, as in DFP and FA, resulted in a drastic decrease of the hydroxyl radical scavenging activity of phenolic compounds (*31*, *32*). Additionally, CFP including both CA and FA moieties showed moderate DPPH, superoxide anion, and hydroxyl radical scavenging activities between DFP and DCP. Thus, it is understandable on the basis of these results that DCP and DFP, which possessed the partial active chemical

**Table 3.** Inhibitory Activity ($IC_{50}$) of Three Major Polyamine Conjugates Isolated from Corn Bran and Their Related Compounds against Tyrosinase Activity and Melanin Synthesis in B16 Melanoma Cells[a]

| | inhibitory activity ($IC_{50}$, $\mu$M) | | |
|---|---|---|---|
| compound | tyrosinase (L-tyrosine) | tyrosinase (L-DOPA) | melanin (B16 melanoma cell) |
| CA | 463.4 ± 16.4 a | 1951.8 ± 157.5 a | 5773.2 ± 362.5 a |
| FA | 277.6 ± 8.2 c | | 4864.4 ± 237.5 b |
| CFP | 233.6 ± 20.9 d | 921.5 ± 36.0 b | 1257.6 ± 123.9 d |
| DFP | 291.3 ± 18.3 c | | 733.6 ± 103.3 e |
| DCP | 181.7 ± 25.8 e | 1056.7 ± 106.8 b | 3169.5 ± 212.2 c |
| L-ascorbic acid[b] | 331.5 ± 2.7 b | | |
| kojic acid[b] | | 7.6 ± 0.9 c | |
| arbutin[b] | | | 1110.4 ± 132.8 d |

[a] $IC_{50}$ represents the concentration of a compound required for 50% inhibition of mushroom tyrosinase and melanin synthesis in B16 melanoma cells. Data represent means ± SD of triplicate determination. Values with the different superscript letters in each column are significantly different at $P < 0.05$. [b] L-Ascorbic acid, kojic acid, and arbutin were used as positive references.

structure of hydroxycinnamic acid bisamide, may play an important role in inhibition of superoxide anion and hydroxyl radicals.

The superoxide and hydroxyl radicals of reactive oxygen species are known to have a high and indiscriminate activity and cause severe oxidative damage to susceptible biomolecules (*3*, *4*). However, this damage can be modulated by dietary antioxidants acting as radical scavengers. Previously, antioxidant phenolic compounds in corn steep liquor bo, such as CA, FA, and their derivatives, were reported to play a beneficial role against oxidative damage (*23*). The current study also revealed that three polyamine conjugates in corn bran, including DCP, CFP, and DFP, acted as superoxide anion and hydroxyl radical scavengers. Thus, these results suggest that corn bran, together with corn steep liquor, obtained as a byproduct in the process of manufacturing starch from corn, may both be useful as potential sources of phenolic antioxidants as radical scavenger. This is the first report on the radical scavenging activity of polyamine conjugates in corn bran.

**Inhibition of Tyrosinase Activity.** Inhibitory effects of three polyamine conjugates and related two hydroxycinnamic acids on mushroom tyrosinase activity using L-tyrosine and L-DOPA as the substrates were determined, and their $IC_{50}$ values are shown in **Table 3**. When L-tyrosine was used as a substrate, DCP ($IC_{50}$ = 181.73 $\mu$M) showed the strongest inhibitory activity, and its inhibitory activity was higher than L-ascorbic acid ($IC_{50}$ = 331.48 $\mu$M), a well-known monophenolase inhibitor against mushroom tyrosinase. FA ($IC_{50}$ = 277.61 $\mu$M) and DFP ($IC_{50}$ = 291.28 $\mu$M) also exhibited considerable inhibitory activity, and their activity was stronger than L-ascorbic acid. However, CA ($IC_{50}$ = 463.38 $\mu$M) showed the lowest activity. On the other hand, when L-DOPA was used as a substrate, all phenolic compounds exhibited much weaker inhibitory activity than kojic acid ($IC_{50}$ = 7.56 $\mu$M), a well-known diphenolase inhibitor against mushroom tyrosinase. Particularly, the maximum inhibition of FA and DFP was not over 50% at concentration range of 50−350 $\mu$M, and CA, CFP, and DCP were less active, contrary to a previous result that FAs and CAs exhibited strong inhibitory activities against L-DOPA oxidation by mushroom tyrosinase (*16*). This discrepancy may be attributable to that fact that different tyrosinase assays were used in the two studies. Thus, the tyrosinase inhibitory effects of three polyamine conjugates against L-DOPA as the substrate was much lower than those against L-tyrosine as the substrate. These results indicate that polyamine conjugates strongly inhibit the pathway from L-tyrosine to dopaquinone on tyrosinase-catalyzed reaction. Many scientific papers have also reported the tyrosinase inhibitory activity of hydroxycinnamic acid derivatives and their conjugated serotonins (*15*, *16*, *33*). They demonstrated that methoxylation and hydroxylation of cinnamic acid derivatives may have played an important role in exhibiting the tyrosinase inhibition activity. However, there is so far no report on the effect of polyamine conjugates from corn bran on tyrosinase activity.

We also examined the inhibitory effects of polyamine conjugates and related hydroxycinnamic acids on melanin synthesis of cultured B16 melanoma cell. Contrary to previous results on tyrosinase inhibition, DFP ($IC_{50}$ = 733.64 $\mu$M) potently inhibited melanin synthesis of B16 melanoma cell, and its activity was stronger than arbutin ($IC_{50}$ = 1110.43 $\mu$M), a well-known tyrosinase inhibitor used as a whitening agent in cosmetics. CFP ($IC_{50}$ = 1257.63 $\mu$M) and DCP ($IC_{50}$ = 3169.45 $\mu$M) also exhibited considerable inhibitory activity, but two hydroxycinnamic acids, FA ($IC_{50}$ = 4864.42 $\mu$M) and CA ($IC_{50}$ = 5773.23 $\mu$M), showed a lower activity than the three polyamine conjugates. Thus, polyamine conjugates in corn bran were found to have a strong inhibitory effect on melanin synthesis in B16 melanoma cell, together with inhibition of tyrosinase activity. Dooley (*34*) previously speculated that a desirable skin-whitening agent should inhibit the synthesis of melanin in melanosomes by acting specifically to reduce the synthesis or activity of tyrosinase. Therefore, these results suggest that polyamine conjugates in corn bran may be useful as potential candidates for novel skin-whitening agents in cosmetics.

Three polyamine conjugates were isolated and purified from corn bran. These polyamine conjugates have been identified as DCP, CFP, and DFP on the basis of spectroscopic evidence and published data. We previously reported that the DFP and CFP were present in corn bran at levels of about 3 and 1 mg/gram of corn bran, respectively (*18*). The levels of DFP and CFP in corn bran were higher than those of free FAs and CAs (*18*). Because the current study focused on evaluating their antioxidant properties, precise quantification of these compounds was not performed. DFP exhibited considerable free DPPH radical and superoxide radical scavenging activities, whereas DCP exhibited the strongest hydroxyl radical scavenging activity. Meanwhile, DCP and DFP exhibited potent tyrosinase inhibitory activities toward L-tyrosine as the substrate and significantly inhibited melanin synthesis in B16 melanoma cells, respectively. The different properties of the five phenolic compounds on the radical scavenging activities and tyrosinase activities may be caused by the different functional groups in their structures and their different solubility in each bioassay system. These results suggest that the polyamine conjugates in corn bran may be useful as potential radical scavengers capable of controlling reactive oxygen-mediated pathological disorders and as skin-whitening agents in cosmetics. Further study is required to isolate and identify minor polyamine conjugates from corn bran and investigate their antioxidant and antimelanogenic effect conjugates in vitro and in vivo, together with three major polyamine conjugates.

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## NOTE ADDED AFTER ASAP PUBLICATION

The first sentence of the paragraph Inhibition of Melanin Production in B16 Melanoma Cells has been modified from that in the original posting of March 31, 2007. The posting of April 11, 2007, includes the correction.

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